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Chemical assembly of multiple metal cofactors: The heterologously expressed multidomain [FeFe]-hydrogenase from *Megasphaera elsdenii*

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ABSTRACT

[FeFe]-hydrogenases are unique and fascinating enzymes catalyzing the reversible reduction of protons into hydrogen. These metalloenzymes display extremely large catalytic reaction rates at very low overpotential values and are, therefore, studied as potential catalysts for bioelectrodes of electrolyzers and fuel cells. Since they contain multiple metal cofactors whose biosynthesis depends on complex protein machineries, their preparation is difficult. As a consequence still few have been purified to homogeneity allowing spectroscopic and structural characterization. As part of a program aiming at getting easy access to new hydrogenases we report here a methodology based on a purely chemical assembly of their metal cofactors. This methodology is applied to the preparation and characterization of the hydrogenase from the fermentative anaerobic rumen bacterium *Megasphaera elsdenii*, which has only been incompletely characterized in the past.

1. Introduction

[FeFe]-hydrogenases, named HydA, are unique biocatalysts for the interconversion between protons and dihydrogen [1]. These metalloenzymes display extremely large catalytic reaction rates at very low overpotential. As a consequence, they have been well studied from structural and mechanistic perspectives [2–4]. Furthermore they are considered as potential alternative catalysts to noble metals for the development of novel bio-fuel cells and bio-electrolyzers as well as bio-photoelectrochemical cells [5–8]. These green technological electrochemical devices might contribute to solve the energy storage issue linked to the intermittency of renewable energies such as solar and wind energies. A large number of studies have thus been devoted to the investigation of the electrochemical behavior of these enzymes at carbon-based surfaces as well as in combination with semiconducting materials in order to optimize bio-electrodes or bio-photoelectrodes [9,10]. These enzymes display remarkable electrocatalytic activity for proton reduction into H_2 but also suffer from extreme oxygen sensitivity [11–15]. Finally, spectroscopic and structural studies provide a knowledge that is extensively exploited by synthetic chemists for the preparation and development of bioinspired catalysts, mimicking the unique diiron active center of these enzymes [16,17].

[FeFe]-hydrogenases indeed rely, for activity, on a complex inorganic site, consisting of an organometallic dinuclear Fe subunit, named 2Fe-subcluster, in which the two Fe atoms are bridged by an azapropanedithiolate (adt^{2-}) ligand and the coordination sphere is completed by CO and cyanide ligands [18]. This diiron complex is linked to a [4Fe-4S] cluster via a sulfur atom of a cysteine residue. This active site, thus containing 6 Fe atoms and named the "H-cluster", is buried inside the protein but is connected to the surface of the protein, for electron transfer, in most cases via an array of iron-sulfur [FeS] clusters (Fig. 1). Interestingly, the number of accessory [FeS] clusters varies from one enzyme to another. For example, HydA from *Clostridium pasteurianum* [19,20] contains one [2Fe-2S] and three [4Fe-4S] clusters, HydA from *Desulfovibrio desulfuricans* [21] harbors two [4Fe-4S] clusters while HydA from the green algae *Chlamydomonas reinhardtii* [22] has no accessory [FeS] clusters.

Biomolecular studies on [FeFe]-hydrogenases are unfortunately limited by the fact that these enzymes are difficult to prepare. First,

Abbreviations: β-met, β-mercaptoethanol; DTT, dithiothreitol; adt, azadithiolate; DMSO, dimethylsulfoxide; EPR, electron paramagnetic resonance; FTIR, Fourier transform infrared; TOF, turnover frequency; Tris, tris(hydroxymethyl)-aminomethane; GC, gas chromatography; SEC, size exclusion chromatography; MeHydA, [FeFe]-hydrogenase from *Megasphaera elsdenii*; CrHydA1, [FeFe]-hydrogenase from *Chlamydomonas reinhardtii*; CpHydA, [FeFe]-hydrogenase from *Clostridium pasteurianum*; DdH, [FeFe]-hydrogenase from *Desulfovibrio desulfuricans*; CaHydA, [FeFe]-hydrogenase from *Clostridium acetobutylicum*; apo-MeHydA, MeHydA without FeS cluters; FeS-MeHydA, MeHydA reconstituted with [4Fe4S] clusters; Holo-MeHydA, FeS-MeHydA maturated with $[Fe_2(adt)(CO)_4(CN)_2]^{2-}$; H_{ox}, oxidized state of [FeFe]-hydrogenase; H_{ox}-CO, CO-inhibited oxidized state of [FeFe]-hydrogenase; H_{red}, reduced state of [FeFe]-hydrogenase; H_{sred}, superreduced state of [FeFe]-hydrogenase; CW, continuous wave; FID, free induction decay.

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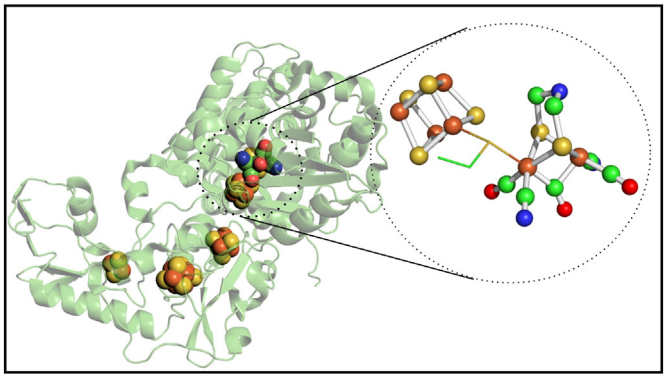

Fig. 1. X ray structure of CpHydA with its protein surrounding, PDB 4XDC [28]. CpHydA has 1 [2Fe2S], 2 [4Fe4S] clusters and the H-cluster depicted in the circle. The picture is designed using the program PYMOL. Fe atoms are depicted in brown, S in yellow, C in green, N in blue and O in red.

they have to be purified and manipulated only under strict anaerobiosis. Second, their maturation, the process by which the [FeS] clusters and the 2Fe-subcluster are synthesized and assembled, depends on complex and specific protein machineries. In particular, the system involved in the biosynthesis of the 2Fe-subcluster is still incompletely characterized [23]. Three enzymes are participating in the biosynthetic pathway. HydG and HydE are S-adenosyl-L-methionine enzymes. HydG has been shown to produce an {Fe-$(CO)_2(CN)$} synthon that is the first intermediate towards the 2Fe-subcluster [24]. HydE is proposed to participate in the synthesis of the adt^{2-} ligand through an as yet unknown mechanism. HydF, is a GTPase that binds a [4Fe-4S] cluster and serves as a scaffold/carrier protein for assembly of a precursor of the 2Fe-subcluster of HydA. Transfer of the latter to HydA, containing the [Fe-S] clusters but lacking the 2Fe-subcluster, produces a fully active hydrogenase. The three proteins are absent from *Escherichia coli* which is the bacterial expressing system, very widely used for recombinant protein preparation [1,25]. As a consequence few [FeFe]-hydrogenases have been purified to homogeneity and only three have been structurally characterized [19–22]. There is thus a need to get easier access to more members of that class of enzymes.

Recently, we discovered that recombinant [FeFe]-hydrogenases can be prepared anaerobically in an inactive form containing only the [FeS] clusters and subsequently fully maturated by reaction with the synthetic $[Fe_2(adt)(CO)_4(CN)_2]^{2-}$ biomimetic complex [26,27]. The resulting active site has been shown to display EPR and FTIR characteristics identical to those of the naturally maturated enzyme, as illustrated in the case of CrHydA1 from *Chlamydomonas reinhardtii*. In case of HydA from *Clostridium pasteurianum* the definitive confirmation that the chemically synthesized active site was identical to the biosynthesized one came from the observation of a remarkable identity of the two three-dimensional structures [28]. This synthetic maturation methodology has already led to a number of very interesting applications which would have been otherwise unreachable, such as specific labeling of the active site or preparation of artificial hydrogenases [4,29–33].

Here, we go a step further towards the facile preparation of active hydrogenases in large amounts from standard *E. coli* strains lacking the HydEFG machinery. We show that it is sufficient to express the corresponding gene in *E. coli*, purify the apoprotein aerobically and activate it chemically, simply via, first, incorporation of the [FeS] clusters by treatment with iron and sulfide and, second, incorporation of the 2Fe-subunit by reaction with the $[Fe_2(adt)(CO)_4(CN)_2]^{2-}$ complex, anaerobically. This methodology is illustrated here with the preparation of HydA from the fermentative anaerobic rumen bacterium *Megasphaera elsdenii*. This enzyme, named MeHydA hereafter, has been previously isolated from a *M. elsdenii* strain but only partially characterized in the 1980s, when the structure of these enzymes was not yet known [34–37]. In this report, we provide a straightforward method for preparing a highly active hydrogenase. Furthermore we provide the first full characterization of MeHydA using EPR and FTIR spectroscopy.

Finally, up to now only two [FeFe] hydrogenases, HydA from *Desulfovibrio desulfuricans* (DdH) and from *C. reinhardtii* (CrHydA1), have been thoroughly studied for their redox behavior [38–40]. The observed redox states differ from each other substantially. The bacterial enzyme DdH can be prepared under aerobic conditions and isolated in the inactive air-stable H_{ox}^{air} state. Reductive activation leads to an intermediate redox state H_{trans} which converts into the active oxidized state called H_{ox}. While the H_{ox}^{air} and H_{trans} are not observed in any other [FeFe] hydrogenase, the H_{ox} state and its CO inhibited version H_{ox}-CO are found in all [FeFe] hydrogenases (Table 2). One electron reduction of H_{ox} (−400 mV NHE, pH 8.0) provides the reduced state H_{red}. Further reduction of DdH (−560 mV NHE, pH 8.0) is incomplete and leads irreversibly to a weakly populated so called "super-reduced" H_{sred} state [38]. In contrast, in CrHydA1, lacking accessory [FeS] clusters, reduction to the super-reduced state (−460 mV NHE, pH 8.0) is complete and reversible [39,40]. In the current study, we also report, for the first time, the redox behavior of MeHydA.

2. Materials and methods

2.1. Expression and purification of apo-MeHydA

TunerDE3pLysS cells transformed with the pT7-7-6HMeHydA plasmid [41] were grown in Terrific Broth medium supplemented with ampicillin and chloramphenicol at 37 °C, until the optical density at 600 nm reached 0.5. Protein synthesis was induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.5 mM. Cells were grown for an additional 5 h with decreasing the temperature to 20 °C to avoid the formation of inclusion bodies. Cells were harvested by centrifugation and stored at −80 °C until use. Cells were resuspended in Tris Buffer 50 mM pH 8.0 containing 5% *v*/v glycerol, 1% *v*/v Triton, 1 mM Dithiothreitol (DTT) and 5 mM β-Mercaptoethanol (β-met) and discontinuously sonicated for 10–12 min. Cellular extracts were centrifuged 1 h at 193,000 *g* leading to a soluble fraction of HydA.

The first chromatographic step was performed on a His Trap column (GE-healthcare) equilibrated with 50 mM Tris–Cl pH 8.0, 300 mM NaCl, 10% *v*/v glycerol, 5 mM β-met, 1 mM DTT. After loading, the column was extensively washed with 10 column volumes of 50 mM Tris–HCl pH 8.0, 300 mM NaCl, 10% *v*/v glycerol, 5 mM β-met, 1 mM DTT, 10 mM imidazole then eluted with a linear gradient of buffer A/buffer A supplemented with 500 mM imidazole. Elution fractions containing 6H-MeHydA were pooled and a pure and homogeneous protein was obtained after a gel filtration step with a Superdex S200 26–600 equilibrated in 50 mM Tris–HCl pH 8.0, 300 mM NaCl, 10% glycerol, 5 mM DTT. Protein concentrations were determined with the Bradford assay (Bio-Rad), using bovine serum albumin as a standard. The oligomeric state of the apo- and reconstituted proteins was determined in anaerobiosis via analytical gel filtration using a Superdex S200 10/300 column equilibrated in 50 mM Tris-HCl buffer pH 8.0, 300 mM NaCl, 10% *v*/v glycerol, 5 mM DTT. To estimate the molecular weight of apo-MeHydA, static light scattering measurements were performed in batch mode in a quartz cuvette on a Zetasizer ZSP (Malvern) using toluene as a standard. The measurements were done from apo-MeHydA freshly purified on a S200 10/300 GL column. The protein concentration was estimated with the Bradford assay. A dn/dc of 0.187 at 633 nm, calculated from the protein sequence using the program SEDFIT was used [42]. The molecular weight was extracted from the intercept of the linear fit with the Zimm equation for an isotropic scatterer, $Kc/R_\theta = 1 / M + 2A_2c$, of the Debye Plot. K is an optical constant, c is the concentration of the analyte, R_θ is the Rayleigh ratio, M is the molecular weight and A_2 the second virial coefficient.